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ACTIVATION OF MACROPHAGES IN VIVO BY GLYCAN LNFPIII

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ACTIVATION OF MACROPHAGES IN VIVO BY GLYCAN LNFPIII

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ABSTRACT

LNFPIII, a schistosome glycan, has been shown to induce an anti-inflammatory response *in vivo* and *in vitro*. In this study we demonstrate the effects that LNFPIII has on macrophages using mouse models. When mice are injected with LNFPIII-dex the production of inflammatory cytokine IL-6 is significantly decreased, compared to uninjected and dextran injected mice. We also found that macrophages from LNFPIII injected mice show signs of alternative activation, including the upregulation of Arginase-1. LNFPIII-activated macrophages also express higher levels of the surface markers CD80 and PDL-1. Lastly, LNFPIII- activated macrophages were able to prime T cells *in vitro* as seen by a decrease in the production of IFN-γ and IL-13.

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank my principal investigator, Dr. Donald Harn, for giving me the opportunity to come into the lab and work with you over multiple summers and into the Fall. I would have never thought that a summer internship two years ago would lead to such a fascinating senior project and a wonderful experience. Your willingness to allow me to remain in the lab and work on my research project has given me opportunities that I would not have had otherwise. I will be forever grateful for the amount that I have learned and the experience that I have gained from working with you. I would also like to thank Olga Atochina for working directly with me on my project and taking the time to edit my drafts. I enjoy working with you every day, and I thank you for helping me expand my knowledge and skills. I would also like to thank both Jasmine MacDonald and Akram Da'Dara for providing answers to my many questions, and for help around the lab when I needed it. I would also like to thank my advisor, Dave Adams, for helping me initiate this project, and help with editing the paper. I would not have had this opportunity without your help in planning my time at WPI.

BACKGROUND

The human body is constantly subjected to infectious agents, however, usually the body is able to fight these infections off. The immune system acts as the body's defense against a variety of pathogens. There are many mechanisms that allow the immune system to fight of such a wide range of diseases. The two main components of the immune system are the innate and adaptive systems.

Innate Immune System

The innate immune system acts as the body's first line of defense against a pathogen. This response is non-specific and can react to any pathogen that enters the body. This innate response uses a plethora of cells to recognize initial infection by foreign organisms (Janeway and Travers, 1996). Natural killer cells (NKs) are able to recognize antibodies on the surface of virus infected cells or tumor cells and kill them (Janeway and Travers, 1996). They can also kill any cell not viewed as "self", not expressing MHC type receptors. Basophils and eosinophils are commonly associated with parasitic infections. Upon activation, basophils release histamine that is important in allergic responses, and eosinophils release toxins that attack parasites (Janeway and Travers, 1996). Macrophages and neutrophils are able to recognize and bind to receptors on foreign invaders, leading to the secretion of cytokines. Cytokines allow cells to communicate with one another, and often lead to an inflammatory immune response (Roitt et al., 2006). The innate immune system is fast acting but it displays no immunological memory and does not respond faster with a second infection. Further

activation of the adaptive immune system is needed to increase protection (Kimball, 2007).

Adaptive Immune System

Unlike the innate immune system, the adaptive immune system is activated four to five days after infection (Janeway and Travers, 1996). In this response, lymphocytes (T cells and B cells) react only to specific foreign antigens (Roitt et al., 2006). The adaptive immune system also displays immunological memory so that a pathogen will not infect the body twice (Janeway and Travers, 1996). The innate and adaptive immune systems must work together in order to develop an appropriate immune response. The link between innate and adaptive immunity is provided by antigen presenting cells.

Antigen Presenting Cells

Antigen presenting cells (APC's), as representatives of the innate immune system, provide the secondary signal needed to induce an adaptive immune response by T cell activation (Janeway and Travers, 1996). The three types of APC's are macrophages, dendritic cells (DC's) and B cells. Since T cells are unable to recognize antigens directly, APC's are able to internalize antigens, process them, and present them to T cells in a recognizable form (Figure 1) (Roitt et al., 2006). APCs internalize antigens either by phagocytosis or recognition of Pathogen Associated Molecular Patterns (PAMPS), such as LPS, by Toll-Like Receptors (TLR's) (Thomas and Harn, 2004). The antigen is processed inside of the cell then presented to the T cell in one of two ways. In the first

way, T cell Receptors (TCR) recognize the Major Histocompatability Complex (MHC), which presents the antigen (peptide) (Kimball, 2007).

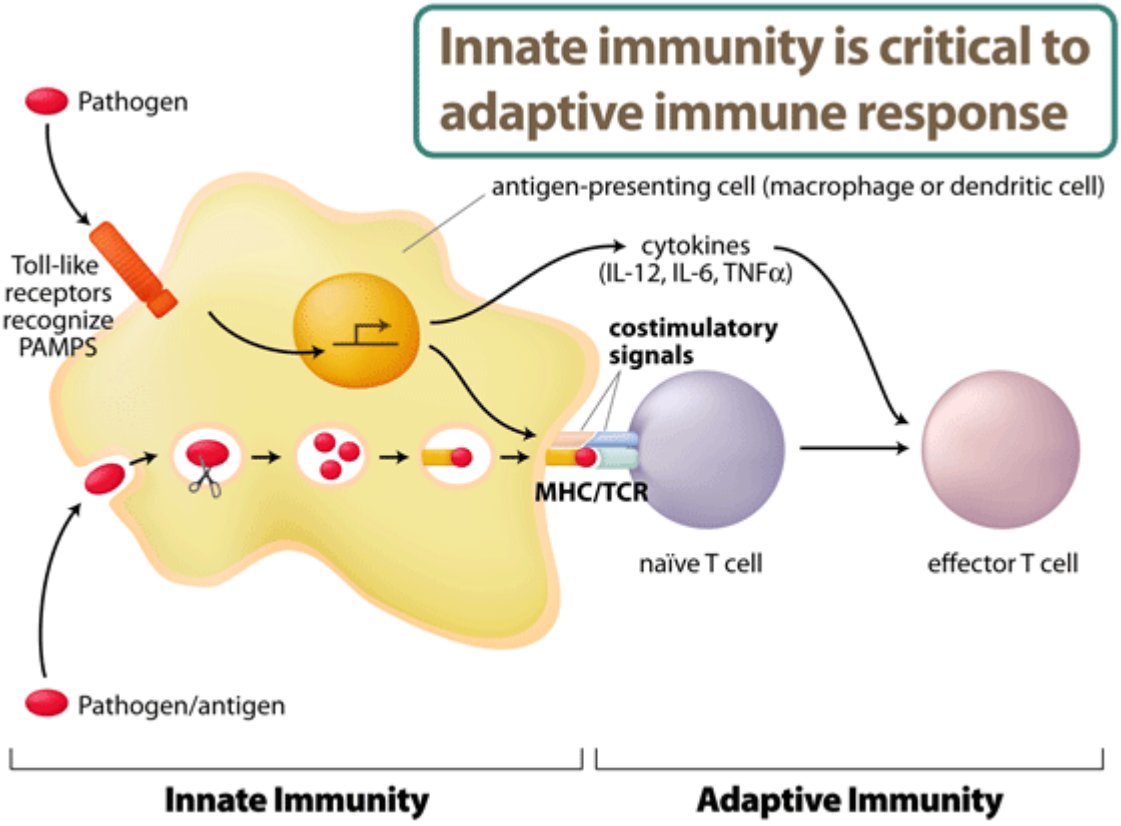


Figure 1. The Link Between Innate and Adaptive Immunity. Antigen presenting cells (yellow), representing the innate immune system, process and present foreign antigens to naïve T cells (blue), representing the adaptive immune system. (Dana Farber Cancer Institute, 2007).

In the second way, costimulatory molecules act as a second signal (Figure 2). Receptors on the surface of the T cell recognize certain co-stimulatory molecules expressed on the APC's surface. This can be seen in the figure with the CD 28/B7 complex, and also includes the CD40/CD40L complex, the ICOS/ICOS-L complex, and others. Naive T Cells can mature into effector T cells such as CD4+ T Helper Cells (Th1 or Th2 type) or CD8+ Cytotoxic T cells (Kimball, 2007).

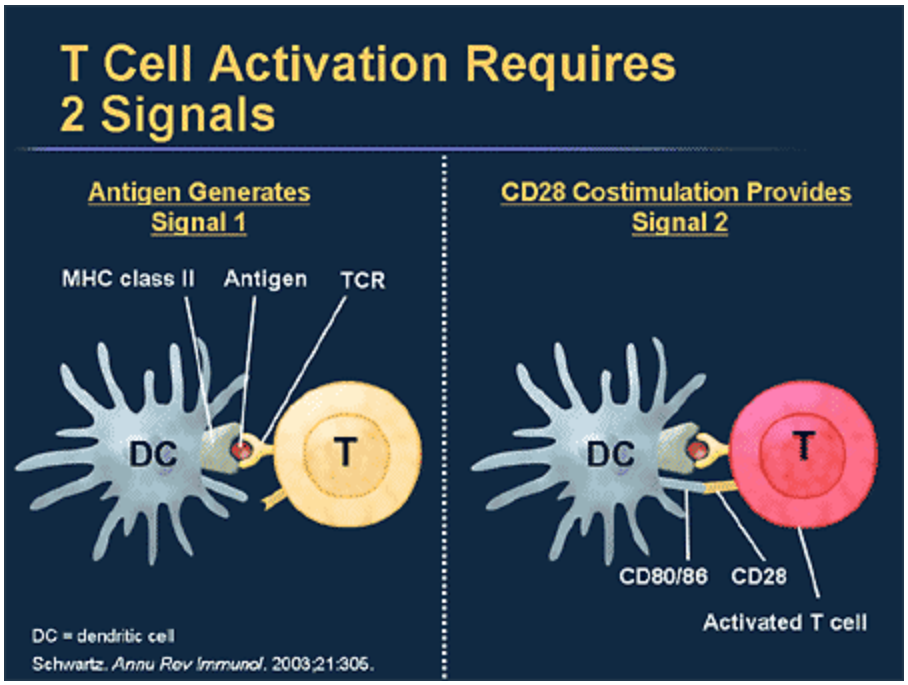


Figure 2. The Use of Costimulatory Molecules by Antigen Presenting Cells. In this instance the co-stimulatory molecules are represented by the CD28/CD80/86 interaction shown in the lower right (Kochar 2006).

T Helper Cell Immune Responses

There are two types of immune responses led by mature, CD4+ T helper cells: the Th1 type induces a pro-inflammatory response by producing mediators such as interferon gamma (IFN-γ), TNF-α, Interleukin – 12 (IL-12), and IL-2. The Th2 type induces an anti-inflammatory response by producing IL-4, IL-5, IL-13, and IL-10 (Thomas and Harn, 2004). It has been shown that the binding of PAMPs such as LPS to Toll-Like Receptors (TLRs) activates dendritic cells and macrophages to induce these differential immune responses (Medzhitov, 2001). Much is known about the activation of DC1s (DCs that induce a Th1 response), but until recently, little was known about the activation of DC2s.

Classically and Alternatively Activated Macrophages

In response to pathogens, two types of macrophages can arise. Classically activated macrophages (CAMph) develop in response to Th1 type mediators such as IFN-γ and TNF (Edwards et al, 2006) and most other antigens including LPS and CpG. Following the binding of PAMPS to CAMph surface receptors and the induction of signal transduction events, LPS and other PAMPS are taken into the cell, where they are degraded (Edwards et.al 2006). A CAMph response is characterized by an increase in iNOS activity in macrophages (De'Broski et al., 2004). The secretion of nitrogen by these cells kills pathogens and induces an inflammatory response. However, in some cases, the inflammatory response can lead to apoptosis and severe tissue damage (Edwards et al 2006). CAMph have been shown to respond to immunodeficiency syndromes such as AIDS as well as autoimmune diseases (Edwards et al., 2006). Upon activation, CAMph express many surface markers such as CD80, CD86, MHC II, ICOS-L and PD-L1 (Kuipers et al., 2006). They are also known to secrete cytokines such as IL-6, TNF, and IL-1 (Gordon, 2003).

In contrast to CAMph, alternatively activated macrophages (AAMph) develop in response to the Th2 type cytokines, IL-4 and IL-13 (Gordon, 2003). This response is commonly seen in allergic response to parasite infection and asthma. Alternative activation of macrophages is characterized by the upregulation of Arginase-1, which is a key factor in granuloma formation (Gordon, 2003). It is also thought to promote growth of the extracellular matrix (Martinez et al., 2008). Activation of AAMph leads to an anti-inflammatory response, while promoting cell proliferation. They are able to activate T cells by cell-cell interaction and by cytokine production. Some studies have shown

AAMph to have a deactivating effect on T cells (Gordon, 2003). The surface markers CD80 and PDL-1 have been associated with macrophage activation and maturation (Martin-Orozco et al., 2006; Keir et al., 2006; Nakanishi et al., 2007). Although the topic is still under debate, it is thought that these markers act as inhibitors to a T cell response (Liang et al., 2003; Zhao et al., 2007; Kuipers et al., 2006). Other markers such as YM-1, FIZZ-1, MGL-1, and the mannose receptor (MMR) have also been associated with alternative activation (Raes et al., 2002). The secretion phenotype of AAMph cells is still unidentified. Figure 3 compares classical versus alternative macrophage activations.

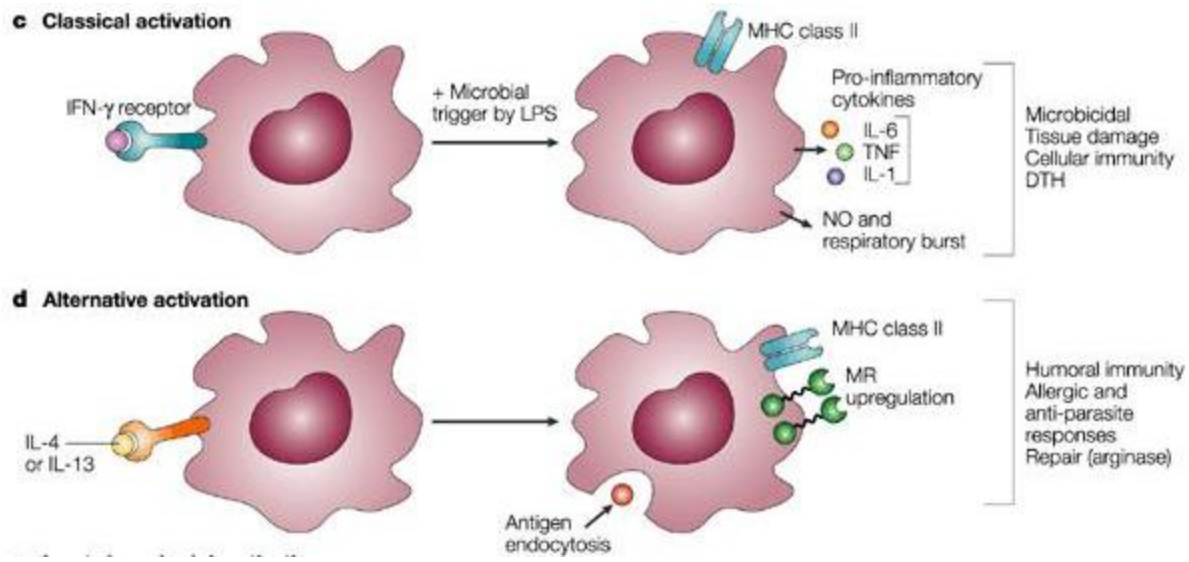


Figure 3. Comparison of Classical versus Alternative Activated Macrophages. Picture Adapted from Gordon, 2003)

Lacto-N-Fucopentaose III

Studies have shown that helminth parasite infection leads to a Th2 type, anti-inflammatory response (Okano et al., 1999; Okano et al., 2001; Thomas et al., 2003; Thomas and Harn, 2004). It was shown that individuals infected with the schistosoma

parasite are least likely to develop atopic diseases (Yazdanbakhsh et al., 2002; Thomas and Harn, 2004;). It was also shown that antibodies developed against the parasite contain the Lewis^x trisaccharide, which is commonly found on schistosome eggs (Harrison and Ridley, 1975; Nash, 1978; Nash et al., 1978; Okano et al., 1999; Thomas and Harn, 2004). It has been shown that Lewis^x containing sugars bind to C-type Lectin (CTL) receptors, such as DC-SIGN, the mannose receptor (MMR), and MGL, that are expressed on dendritic cells and macrophages (Van Leimpt et al., 2006).

The Th2 immune response was found to be driven by schistosome glycans (Thomas and Harn, 2004). One glycan of interest is the polysaccharide Lacto-N-fucopentaose III (LNFPIII). LNFPIII contains the Lewis^x trisaccharide and can be found during natural immunosuppressive situations such as pregnancy and cancer, and also in human breast milk, and in the urine of pregnant women (Erney et al., 2001; Kedler et al., 2001; Pfeninger et al., 2001; Thomas and Harn, 2004). Other glycans, such as LNnT, that do not contain the Lewis^x trisaccharide, do not display the Th2 type response, suggesting that the Lewis^x trisaccharide plays an important role in the Th2 pathway (Thomas and Harn, 2004). LNFPIII has been shown to aid in the prevention of some autoimmune diseases such as psoriasis (Atochina and Harn, 2006), diabetes (Maron et al., 1998), and colitis (Elliot et al., 2003).

It has been shown that LNFPIII induces a Th2 type response *in vitro*, however the mechanism by which LNFPIII activates macrophages *in vivo* is unclear. Little is known about the profile of cytokine secretion induced by LNFPIII. The profile of LNFPIII induction of alternative activation is also unknown. The understanding of how LNFPIII works *in vivo* is crucial to the use of this sugar for the treatment of diseases.

In our studies, LNFPIII (βGal-[1-4]-(αFuc-[1-3])-βGlcNAc-[1-3]-βGal-[1-4]-Glc) was conjugated to a 40 kDa molecule of dextran (Dex), which is used as a carrier molecule (Figure 4). The glycoconjugate consisted of ~12 LNFPIII molecules conjugated to a 40 kDa molecule of Dex.

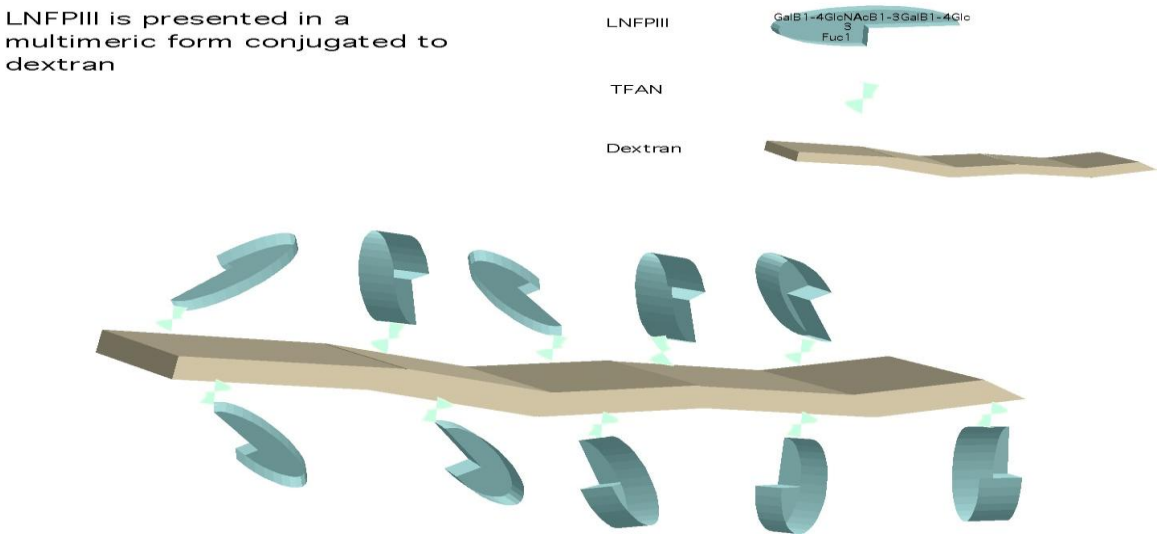


Figure 4. Diagram of Lacto-N-fucopentaose III Structure (Thomas and Harn, 2003).

PROJECT PURPOSE

The mechanism by which LNFPIII leads to a Th2 type response is still unclear. It has been shown that LNFPIII induces a Th2 type anti-inflammatory response *in vitro*. However, the role that LNFPIII plays in activating macrophages *in vivo* is not known. The purpose of this study is to demonstrate the effects that LNFPIII has on macrophages using mouse models. First, we investigated the effects of LNFPIII on macrophage production of cytokines and chemokines by using a Mouse Cytokine Array and by ELISA assay. Next, we investigated signs of alternatively activated macrophages including Arginase-1 by western blot, and surface markers CD80 and PD-L1 by FACS analysis. Lastly, we investigated LNFPIII's ability to activate T cells *in vitro* by co-culture of PECs and T cells.

METHODS

Carbohydrates

The polysaccharide Lacto-N-fucopentaose (LNFPIII) was obtained from Dr. David Norberg, Sweden. LNFPIII (βGal-[1-4]-(αFuc-[1-3])-βGlcNAc-[1-3]-βGal-[1-4]-Glc) was conjugated to a 40 kDa molecule of dextran (Dex). The glycoconjugate consisted of ~12 LNFPIII molecules conjugated to a 40 kDa molecule of Dex.

Mice

Six to eight week old, female, BALB/c, Black/6, DC Sign $^{-/-}$, Gal 1/3 $^{-/-}$, and DO11.10 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and were maintained under pathogen-free conditions at the Harvard School of Public Health animal facility. The mice were injected intraperitoneally with 100 µg of LNFPIII-Dex in 200 µl of PBS for 24-48 hours.

Isolation of Peritoneal Cells and Splenocytes

Approximately 24 hours post injection with LNFPIII-dextran or dextran, mice were euthanized by CO_2 inhalation. Peritoneal endothelial cells (PECs) were collected by lavage under sterile conditions by injecting 5 ml of PBS into the peritoneal cavity.

The spleen was extracted from DO.11.10 mice. Cells were collected in 5 ml PBS and lysed in 1 ml Boyle's solution. CD4+ T cells were collected by MACS purification (Mitenyi Biotech). Cells were resuspended in MACS buffer and incubated with CD4 microbeads for 15 minutes at 4°C. CD4+ cells were separated magnetically using a MACS column and separator.

Cell Culture

PECs were cultured at an initial density of 1.0 X 10^6 cells/ml in RPMI 1640 medium (Invitrogen), supplemented with 10% Fetal Bovine Serum (HyClone, Logan UT), 100 U/ml streptomycin, 100 U/ml penicillin, 2 mM glutamine (Sigma-Aldrich) and 0.05 mM 2-Mercaptoethanol (Gibco). Cells were cultured in 24 or 48 well plates for 48 hours at 37°C. Supernatants were collected for analysis.

Macrophage-T cell co-culture

PECs were plated at a concentration of 0.5 X 10^6 cells/ml in 0.5 ml of complete media in 48 well plates for 1 hour at 37°C with 1000 or 100 nM of OVA peptide (OVA 223-239 peptide; Sigma-Genosys, Woodlands, TX). Splenocytes from DO.11.10 mice were collected and added to the PEC culture in the same volume and concentration for 72 and 96 hours. Supernatant was collected for analysis by ELISA.

ELISA

The levels of TGF-β, IL-13, IL-6, and IFN-γ (BD Biosciences) were determined by capture ELISA according to the manufacturer's protocol. Briefly, capture antibodies were diluted in the appropriate antibody diluents. 96 well plates (Nalge Nunc International) were coated with capture antibody at 0.5 μg/ml, 1 μg/ml, 2 μg/ml, and 4 μg/ml respectively and incubated overnight at 4°C. Plates were washed in buffer containing PBS and 0.05% Tween-20. The plates were then blocked in buffer containing 1% BSA in PBS for 1 hour and then washed. Plates were incubated with samples and standards for 2 hours at room temperature or overnight and then washed. Next, the plates were incubated in secondary biotinylated antibodies at 0.5 ng/ml, 1 μg/ml, 2 μg/ml and 4

μg/ml, respectively, for 2 hours at room temperature and then washed. Lastly, the plates were incubated in streptavidin HRP (1:2000 dilution, R&D systems) for 1 hour at room temperature and washed. The samples were developed with TMB substrate solution (Kirkegaard and Perry Laboratories), and the reaction was stopped with 5% phosphoric acid. The optical density was read at 450 nm on a Spectramax 190 using softmax PRO 30 alias software. Cytokine concentration was calculated based on the standard curve.

Cytokine Array

The levels of 62 soluble protein molecules were determined by a Mouse Cytokine Antibody Array Kit (RayBiotech). Membranes were blocked in blocking buffer, provided by the kit for 30 minutes at room temperature. The membranes were then incubated with samples overnight at 4°C. Next, the membranes were washed 5x5 minutes with wash buffer. The membranes were incubated in primary antibody for 2 hours at room temperature. Membranes were washed and then incubated in HRP for 2 hours. Lastly, the membranes were washed, incubated in detection buffer for 2 minutes, and the image was captured on a Kodak Imager.

Western Blots

Cells were harvested in 100 μl of SDS sample buffer, containing Laemelli sample buffer and 2-ME (Bio Rad). Standard western blot protocol was performed. The samples were run on a 4-20% Tris-HCl gel (Bio Rad) and transferred onto an Immobilon Transfer Membrane (PVDF) (Millipore). The membranes were blocked in 5% milk / 1X TBS-0.1% Tween-20 for 1 hour at room temperature. The membrane was incubated with

Arginase-1 antibody (BD Biosciences), or total ERK antibody (Cell Signaling), overnight at 4°C. The membrane was incubated with anti- mouse IgG antibody (Cell Signaling) and anti rabbit-HRP conjugated antibody (Cells Signaling) respectively for 1 hour at room temperature. The blots were developed with chemiluminescent HRP substrate (Pierce). The image was captured on a Kodak IS440CF Imaging Station with Kodak ID software.

FACs Analysis

Cells were harvested in FACS buffer (PBS containing 0.1% BSA and 0.1% sodium azide) and 0.5 X 10^6 cells in 100 µl were stained for 30 min at 4°C. Cells were stained with F4/80 Antibody (Serotec) – APC labeled, PD-L1-PE, and CD80-PE (BD Biosciences). Cells were washed twice in FACS buffer and analyzed on FACS caliber machine using CellQuest software.

Statistical Analysis

Statistical significance of difference among groups was determined by a Student's t-test. All data are expressed as the mean ± one standard error.

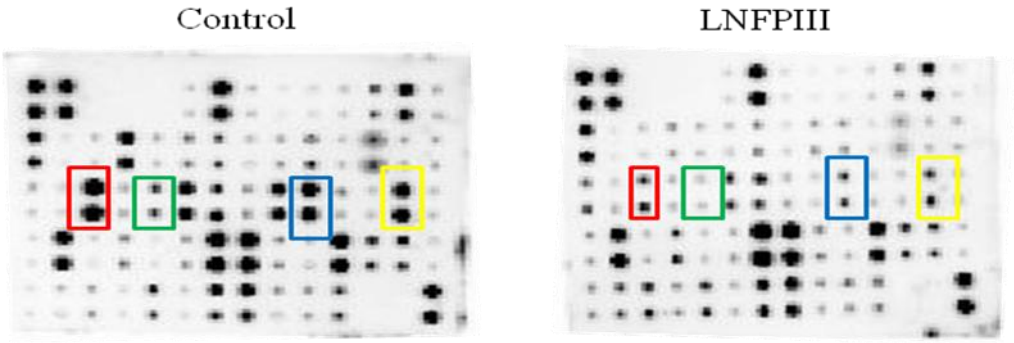
RESULTS

LNFPIII significantly decreases cytokine production

In order to determine the effect of LNFPIII on macrophages *in vivo*, we first investigated the cytokine production of peritoneal endothelial cells (PECs) obtained from mice injected with LNFPIII versus uninjected mice. To do so, we injected Balb/c mice with 100 µg of LNFPIII-dex, a dose previously determined in another study. Twenty-four hours post injection, PECs were collected and plated for 48 hours in complete media without additional stimulation. We then performed a Mouse Cytokine Antibody Array on supernatant from the cultured cells. We found that at least 4 cytokines/chemokines, IL-6, IL-10, KC (chemokine CXCL1), and LIX (chemokine CXCL5), were down regulated in the array of LNFPIII-activated PECs compared to the control PECs from uninjected mice (Figure 5a). After quantitatively analyzing the blots, we determined that IL-6, IL-10, and KC were significantly decreased in PECs from LNFPIII injected mice, whereas the decrease in LIX, a Th-1 type chemokine was not significant (Figure 5b). Surprisingly, we did not find any up regulated molecules among the 62 analyzed cytokine/chemokine/soluble molecules.

To confirm our data from the Mouse Cytokine Antibody Array, we performed an IL-6 and IL-10 combined ELISA using the same supernatants, and found that the level of IL-6 in PECs from LNFPIII-dex injected mice was indeed significantly reduced compared to control mice (Figure 5c). We were not able to confirm IL-10 data by ELISA because of low antibody sensitivity, and anti-KC and anti-LIX antibody are not available for ELISA assay. Thus, using an ELISA we confirmed our Cytokine Array data that IL-6 is significantly down regulated in PECs from LNFPIII injected mice.

A.



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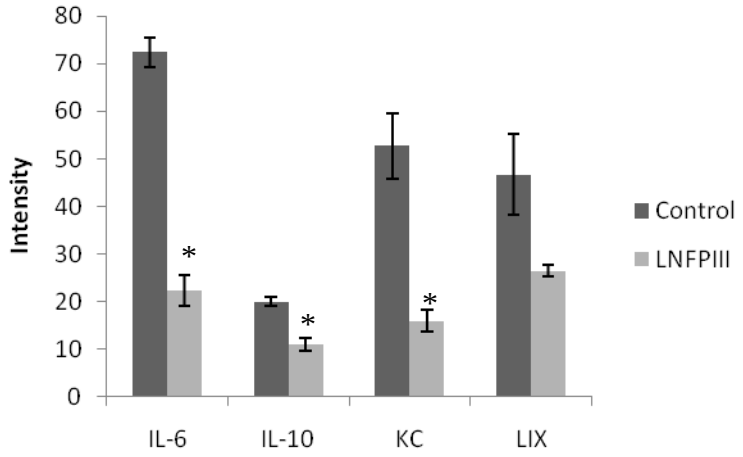

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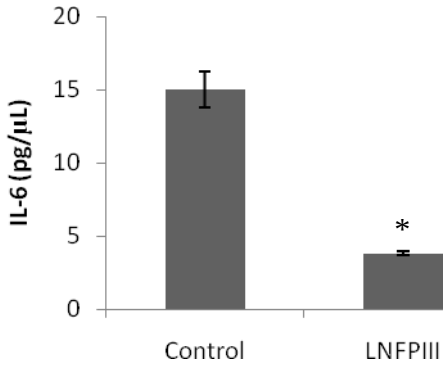
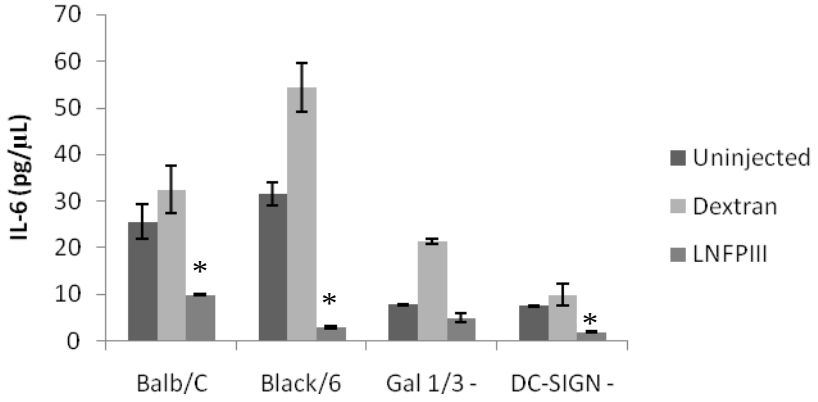


Figure 5. (a) Cytokine Array shows levels of IL-6 (outlined in red), IL-10 (outlined in green), KC (outlined in yellow), and LIX (outlined in blue) proteins in the conditioned medium of 48 hr cultured PEC cells isolated from uninjected mice. (b) Analysis of Cytokine Array shows that IL-6, IL-10, and KC proteins are down regulated (3 significantly) in LNFPIII injected mice. (c) IL-6 ELSIA confirms that IL-6 is significantly down regulated in cultured PECs from LNFPIII injected mice. * $p < 0.05$ for LNFPIII versus control.

Cytokine production of LNFPIII-activated PECs from different strains of mice

We next asked whether the mouse strain is important for the LNFPIII response. We chose to study Balb/c mice, known for a predominantly Th2 type immune response versus Black/6 mice, which are known for a Th1 type response. Since it is known that the Lewis^x trisaccharide binds to C-type Lectin receptors such as Galectin 1 and 3, DC-SIGN, Mannose Receptors, and MGL receptors, we decided to use two strains of knockout mice, Galectin 1/3, and DC SIGN, to determine the effect of LNFPIII on cytokine production. Similar to previous experiments, we injected the mice with LNFPIII for 24 hours and plated peritoneal cells for 48 hours. We collected the supernatant and performed IL-6 and TGF-β ELISAs on these samples. We found that IL-6 was significantly decreased in all samples of PECs from LNFPIII injected mice except for Gal 1/3 knockout mice (Figure 6a). To eliminate the suggestion that LNFPIII caused cells to go through apoptosis, we tested for TGF-β, a cytokine that is known to be present in both Th1 and Th2 type responses. There was no significant increase or decrease in any of the samples (Figure 6b). We conclude that LNFPIII can decrease the production of IL-6 independent of Balb/C or Black/6 genetic background and in DC SIGN-deficient mice, but not in Galectin 1/3 deficient mice.

A.



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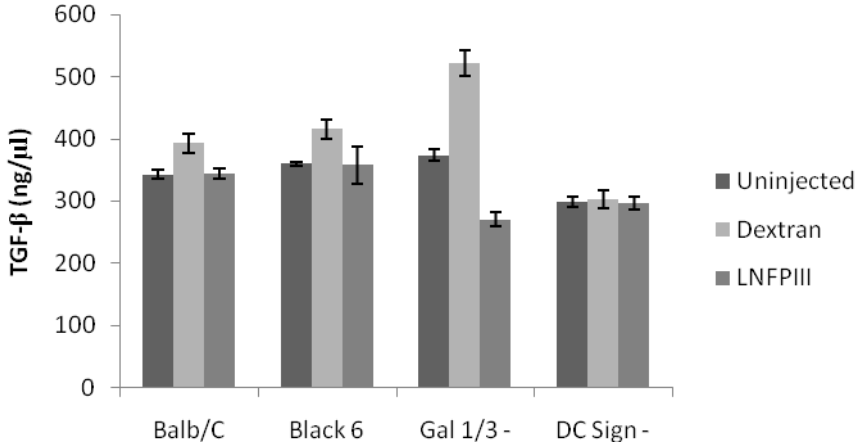


Figure 6. (a) IL-6 is significantly down regulated relative to control in all strains of mice except the Gal 1/3 deficient mice (b) There is no significant decrease in TGF -β in all samples. * $p < 0.05$ for LNFPIII versus control.

Arginase-1 is up regulated in LNFPIII injected mice

We next investigated if macrophages show signs of alternative activation after LNFPIII injection. We injected Balb/c mice with LNFPIII-dex for 24 hours, harvested peritoneal cells, and performed a western blot analysis for Arginase-1 as a marker protein for an alternative activation response. We found that Arginase-1 is significantly up regulated in PECs from LNFPIII injected mice compared to the controls (Figure 7). These results suggest that LNFPIII induces alternative activation of macrophages. Since

there are other known markers for AAMph, such as YM-1, FIZZ-1, MMR, and MGL-1, in the future we will perform the experiments to investigate the alterations of those markers after LNFPIII activation of macrophages using RT-PCR.

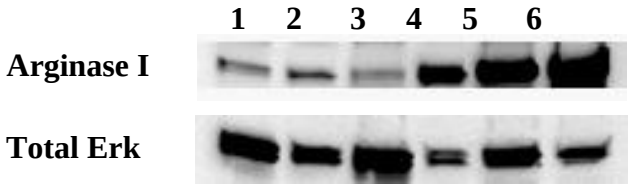
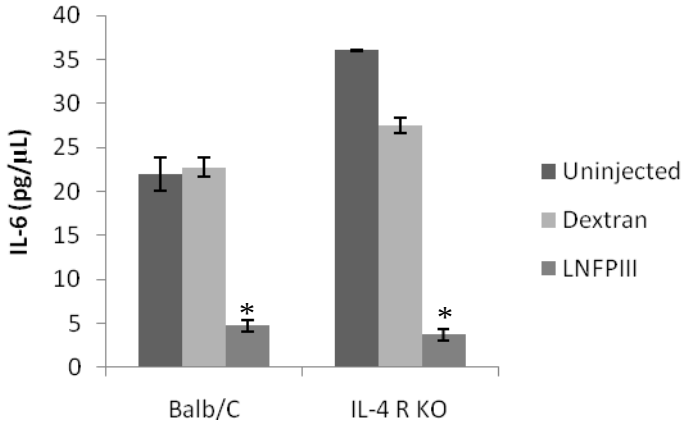


Figure 7. Western blot analysis of Arginase 1 in PECs obtained from uninjected mice (lane 1), 2hrs Dex injected mice (lane 2), 2 hrs LNFPIII-dex injected mice (lane 3), 20 hrs LNFPIII-dex injected mice (lane 4). PECs stimulated with 20 ng/ml rmIL-4 (lane 5) and 1ug/ml LPS+20ng/ml IFN-γ (lane 6) for 20 hrs were used as positive controls. The same blot was stripped and reprobed with anti-Erk antibody as a loading control. The expression of the MAP kinase Erk is activated in response to most PAMPS such as LNFPIII and LPS, leading to transcription.

Activation of PECs by LNFPIII in vivo is IL-4 and IL-13 independent

Alternatively activated macrophages are commonly associated with IL-4 and IL-13 production. CD4+ T cells and eosinophils can secrete IL-4 and IL-13 to activate macrophages. To determine if LNFPIII macrophage activation is IL-4/IL-13 dependent, we used IL-4 Receptor alpha knockout mice (IL-4 Rα KO) because IL-4 and IL-13 share the same receptor on macrophages. We injected IL-4 Rα KO mice with LNFPIII-dex, and dextran for 24 hours and cultured the peritoneal cells for 48 hours *in vitro* without additional stimulation. We collected the supernatant for IL-6 and TGF-β ELISAs. We found no difference in the production of IL-6 or TGF-β for the IL-4 Rα KO mice compared to control Balb/c mice (Figure 8a,b). This suggests that activation of macrophages by LNFPIII in vivo is IL-4 and IL-13 independent.

A.



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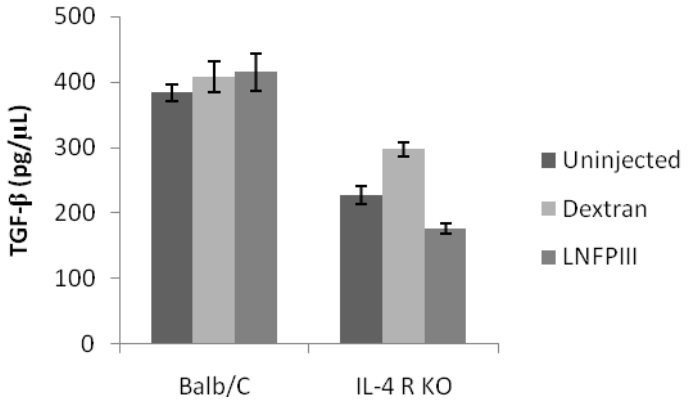

Figure 8 (a) IL-6 is significantly decreased in LNFPIII injected mice for both wild type and IL-4 Rα KO mice. (b). There is no significant decrease in TGF-β produced in either wild type or IL-4 Rα KO mice injected with LNFPIII. * p < 0.05 for LNFPIII versus control.

PECs from mice injected with LNFPIII-dex express CD80 and PD-L1 surface markers

We next investigated the expression of macrophage surface markers that are associated with macrophage activation and maturation. We injected Balb/c mice with LNFPIII-dex for 24 hours, collected PECs, washed and stained them with F4/80 (specific mouse macrophage marker) and PD-L1 and CD80 (markers associated with macrophage activation and maturation). We did the same for IL-4 Rα KO mice to validate our

findings from the cytokine data. We found that both Balb/c wild type and IL-4 Rα KO mice up regulated both PDL-1 and CD80 markers (Figure 9) following LNFPIII treatment. This suggests that LNFPIII injected mice express surface markers associated with alternative activation and also confirms that the IL-4 receptor is not necessary for the function of LNFPIII.

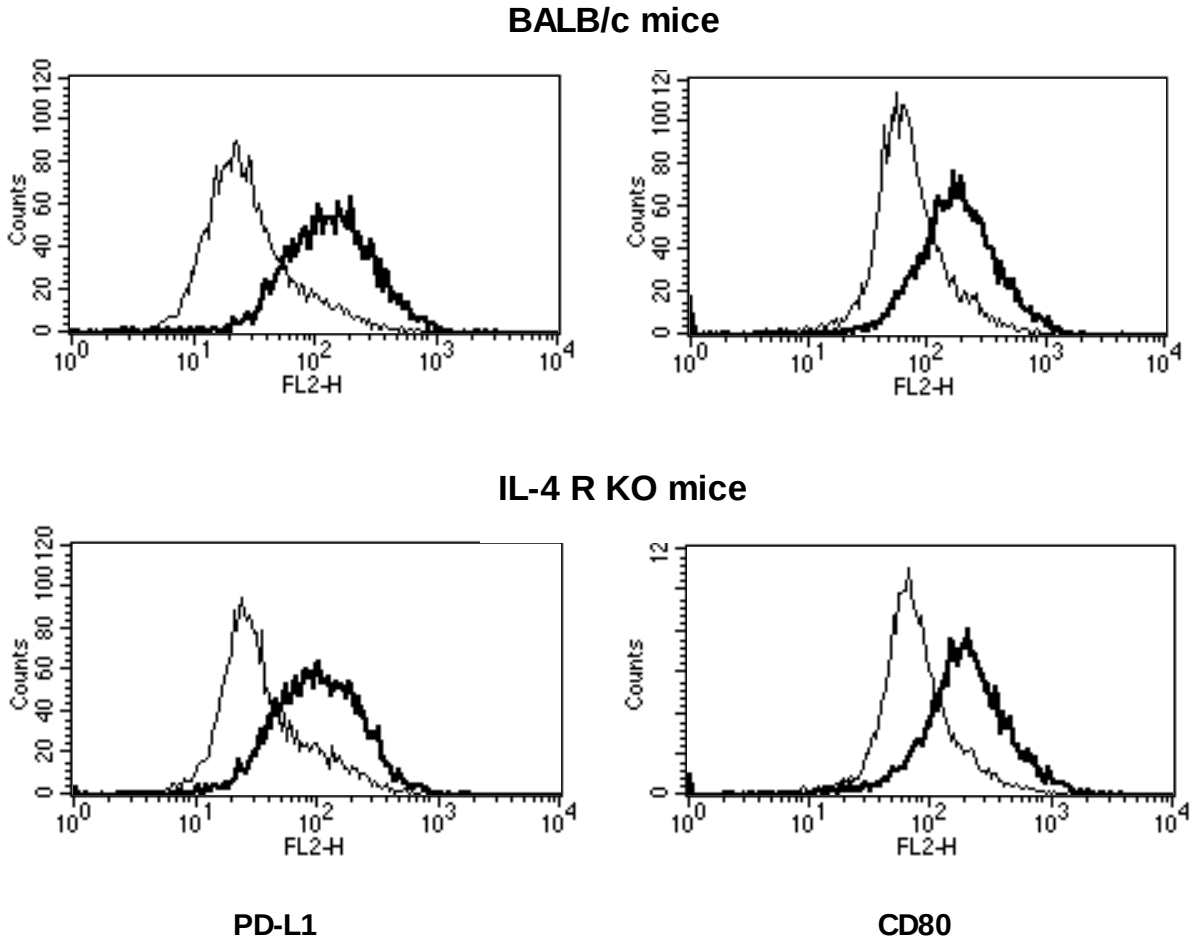

Figure 9. PDL-1 and CD80 expression on F4/80+ macrophages of dextran injected (control) versus LNFPIII injected (bold line) mice.

IFN-γ and IL-13 are significantly decreased in supernatants from co-cultures of T cells and PECs from LNFPIII injected mice

Since we know that macrophages show signs of alternative activation after mice have been injected with LNFPIII, we wanted to determine the effect this sugar has on macrophage's ability to activate T cells *in vitro*. We injected Balb/c mice with LNFPIII–dex for 24 hours and co-cultured the isolated macrophages for 72 or 96 hours with splenocytes from DO.11.10 mice in the ratio of Macrophages to T cells 1:1 and 1:5 in the presence of either 100 or 1000 nM of OVA peptide. We found a dose dependent response of CD4+ T cells to the peptide, where IFN–γ secretion was significantly decreased in co-cultured PECs from LNFPIII injected mice (Figure 10a,b). We also tested a Th-2 type cytokine, IL-13, and found that it is also significantly decreased in the same samples (Figure 10c). We did not find any secretion of IL-4 or IL-5. From this data we were able to determine that LNFPIII is able to activate macrophages *in vivo*, and co-culture of those macrophages with CD4+ T cells from DO.11.10 mice in the presence of OVA peptide induced a decrease in the Th1 type cytokine IFN-γ. Surprisingly, we also found that a Th2 type cytokine, IL-13, was significantly decreased as well. This suggests that LNFPIII activated macrophages are slowing down the production of T cell cytokines.

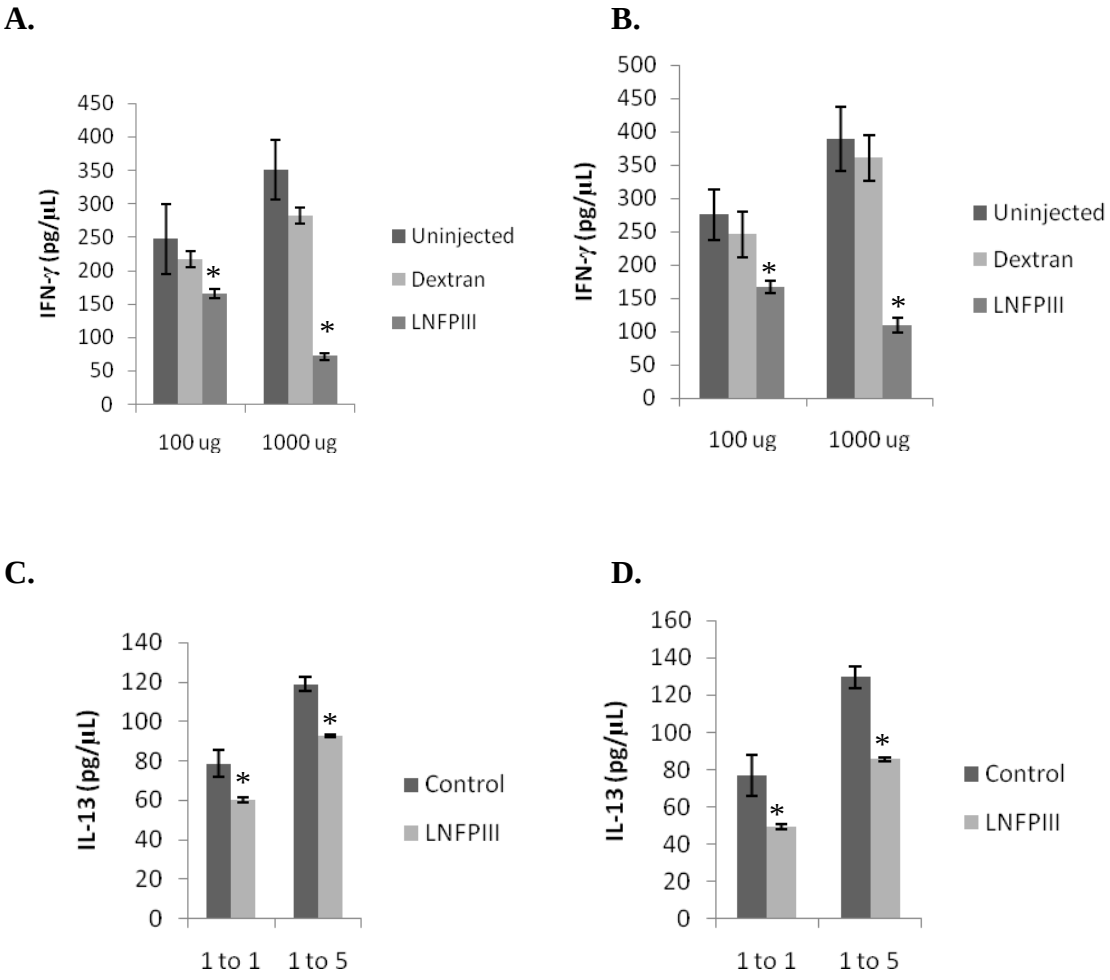

Figure 10. 72 hour (a) and 96 hour (b) macrophage/splenocyte co-culture shows an OVA peptide dose dependent decrease in IFN-γ production of T cells and PECs from mice injected with LNFPIII. Ratio of macrophages to T cells (1:1 and 1:5) has no effect on the dose dependent decrease of IL-13 to 100 nM (c), or 1000 nM (d) of OVA peptide. *p< 0.05 for LNFPIII versus control.

DISCUSSION

Previously, it had been shown that LNFPIII-activated Antigen Presenting Cells (APCs) induce a Th2 type, anti inflammatory response, characterized by a decrease in IFN-γ and an increase in IL-4 production by T cells *in vitro* (Thomas et al., 2003; Thomas and Harn 2004). Atochina and Harn demonstrated that peritoneal macrophages secreted IL-10, PGE2 and TNF-α upon in vitro stimulation with LNFPIII (Atochina and Harn, 2005). In this study, we investigated the mechanism of how LNFPIII activates macrophages *in vivo*.

In response to pathogens, macrophages can become either classically or alternatively activated. Classically-activated macrophages, which have been well characterized, respond to Th1 type mediators such as IFN-γ (Edwards et al, 2006). They are characterized by an increase in iNOS activity (De'Broski et al., 2004). Much less is known about alternatively activated macrophages. It has been shown that alternatively activated macrophages respond to the Th2 type mediators IL-4 and IL-13 (Gordon, 2003). It is well known that macrophages induce T cells to secrete IL-12 and IFN-γ upon stimulation with LPS, a Th1 type promoter (Thomas et al., 2003; Dillon et al., 2004), and IL-4, IL-5, IL-13, and IL-10, in response to LNFPIII (Thomas et al., 2003). Studies have shown secretion of IL-6 in both Th1 and Th2 type responses (Diehl and Rincón, 2002). In our current study, using a Mouse Cytokine Antibody Array, we found that the production of the cytokines, IL-6 and IL-10, and the chemokine KC were decreased in PECs collected from LNFPIII-injected mice compared to controls. KC, or CXCL1, is associated with a group of chemokines that are up regulated in cancer patients (Bièche et al., 2007). LNFPIII has been found to be present in cancer cells and may be acting to

inhibit the production of these chemokines *in vivo*. We confirmed the reduction of IL-6 secretion in PECs from LNFPIII-injected mice by ELISA. Thus we can conclude that LNFPIII has a negative effect on macrophage production of cytokines when injected *in vivo*.

We validated our LNFPIII-induced secretion of IL-6 from PECs, in different strains of mice by ELISA. In this study, we found similar decreased levels of IL-6 in Balb/c mice, known for a predominately Th2 type response, and Black/6 mice, known for a predominately Th1 type response. Our results indicate that LNFPIII is able to function normally even in a predominately inflammatory environment. LNFPIII contains the Lewis^x trisaccharide, which is known to bind to C-type Lectin receptors such as DC-SIGN, MGL-1, MMR, and Galectins (Van Leimpt et al., 2006). It was hypothesized that by eliminating LNFPIII's ability to bind to these receptors, the sugar would not be able to function. We found no difference in the LNFPIII-induced decrease of IL-6 production from PECs of DC-SIGN and Galectin 1/3 knockout mice compared to control mice. These results suggest that when one of those receptors is not present, LNFPIII binds to other available receptors, such as the mannose receptor (MMR) or MGL-1. The levels of TGF-β were not altered by the LNFPIII treatment in any of the mice, confirming that the decrease in cytokine production is not due to apoptosis.

Since a Th2 type response is associated with alternative activation, we investigated whether LNFPIII induces the expression of markers associated with alternative activation such as the upregulation of Arginase-1 (Gordon, 2003; Martin-Orozco et al., 2006; Keir et al., 2006; Nakanishi et al., 2007). We found an upregulation of Arginase-1 in PECs from LNFPIII injected mice compared to control mice. Based on

these results, we conclude that macrophages from LNFPIII injected mice portray the profile of alternative activation. Although other markers have also been associated with alternative activation, such as FIZZ-1, Ym1, and MGL-1, we did not test for these markers since studies have shown that the presentation of these markers on AAMph is not consistent (Raes et al., 2002; Raes et al., 2005; Donnelly et al., 2005; Reece et al., 2006). Future studies will be done using RT-PCR to investigate the expression of these markers in macrophages from LNFPIII injected mice.

Classically-activated macrophages express costimulatory molecules such as CD80, CD86, MHC II, ICOS-L, and PD-L1 on their surface. The expression of these markers has been associated with macrophage activation and maturation (Kuipers et al., 2006). PD-L1 and CD80 have been described as inhibitory molecules (Brown et al., 2003; Ito et al., 2005; Kuipers et al., 2006). Studies have shown that splenic DCs *in vivo* and bone marrow DCs from tumor bearing mice induce the expression of PD-L1 and CD80 (Idoyaga et al., 2007). In our study, we found an upregulation in the expression of the surface markers PD-L1 and CD80 on F4/80+ macrophages from LNFPIII injected mice compared to controls. This data suggests that LNFPIII induces expression of inhibitory molecules, similar to those induced by cancer cells.

The cytokines IL-4 and IL-13 are commonly associated with alternative activation (Gordon, 2003; Anthony et al., 2006). The production of IL-13 has been directly associated with the induction of alternatively activated macrophages characterized by Arginase-1 expression, although Arginase-1 is not always associated with IL-13 (Munder et al., 1998; Prasse et al., 2007). Studies have shown that alternative macrophage activation is dependent upon IL-4 and IL-13 production (De'Broski et al., 2004). Since

IL-4 and IL-13 share the common receptor on macrophages, in the current work we used IL-4Rα KO mice to rule out the role of both cytokines in generating alternatively activated macrophages by LNFPIII *in vivo*. We demonstrate that IL-4 Rα KO display similar profiles of activation (IL-6 production and PDL-1/CD80 expression) as control mice, suggesting that LNFPIII activation is independent of IL-4/IL-13. Although IL-4 and IL-13 are commonly associated with alternative activation, our work suggests that the increase of these cytokines is merely a product of alternative activation and not important for this development in our experimental model.

In vivo, macrophages activate T cells to induce certain immune responses. In order to mimic this, we co-cultured macrophages, with T cells that respond to the OVA peptide. We detected a decrease in the production of IFN-γ from T cells co-cultured with PECS from mice injected with LNFPIII, which is typical in a Th2 type response. However, we also determined a significant decrease in IL-13 secretion (Figure 10), which is typically increased in a Th2 type response (Thomas and Harn, 2004). Since we found an upregulation in the expression of the inhibitory molecules PD-L1 and CD80 on LNFPIII-stimulated macrophages, it is possible that surface markers expressed in alternatively activated macrophages are able to deactivate cytokine production, leading to a Th-2 type response. Keir et al has suggested that PD-L1 acts as an inhibitor to T cell response, however this idea is still under debate (Liang et al., 2003; Kuipers et al., 2006; Zhao et al., 2007). We were not able to detect IL-4 and IL-5 production in the co-cultured samples. A longer co-culture may be necessary in order to detect cytokines that may be produced later in culture.

Alternative activation of macrophages is important for the development of immune responses against allergic diseases and parasitic infections. Our data indicate that LNFPIII induces the alternative activation of macrophages characterized by upregulation of Arginase-1. Furthermore, the data suggests that expression of the surface markers PD-L1 and CD80 leads to the deactivation of T cell production of cytokines as a method of inducing a predominantly Th2 type response. Our work represents findings that add to the understanding of this alternative activation process and will someday make an impact of the treatment of diseases.

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